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(*Z*)-9-NONACOSENE—MAJOR COMPONENT OF THE CONTACT SEX PHEROMONE OF THE BEETLE *Megacyllene caryae*

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Abstract—Male *Megacyllene caryae* (Gahan) (Coleoptera: Cerambycidae) respond to females only after touching them with their antennae, indicating that mate recognition is mediated by a contact sex pheromone. Gas chromatography-mass spectrometry analyses of whole-body solvent extracts of male and female *M. caryae* revealed substantial differences in hydrocarbon profiles, with nearly half of the compounds in the extracts from females being absent from those of males. Biological activities of fractions of crude extracts of females, and reconstructed blends of the most abundant straight-chain (*n*C_{27}, *n*C_{28}, *n*C_{29}), methyl-branched (2Me-C_{26}, 9Me-C_{29}, 11, 13, 15Me-C_{29}), and unsaturated (*Z*9:C_{29}, *Z*13:C_{29}, *Z*14:C_{29}, *Z*13:C_{31}, *Z*14:C_{31}, *Z*15:C_{31}) compounds in extracts of females were tested in arena bioassays, assessing four steps in the mating behavior sequence of males (orientation, arrestment, body alignment, mounting and attempting to couple the genitalia). Males showed limited response to dead females treated with fractions of the crude extract or blends of synthetic straight-chain and methyl-branched alkanes, but responded strongly to the blend of synthetic monoenes. Further trials determined that the complete sequence of mating behaviors, up to and including coupling the genitalia, was elicited by *Z*9:C_{29} alone. *Z*9:C_{29} is a homolog of the contact pheromone (*Z*9:C_{25}) of the congener *M. robiniae* (Förster). Previous work with *M. robiniae* suggested that wipe sampling of cuticular

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hydrocarbons of females by solid phase microextraction yielded a more representative profile of components actually encountered by a male's antennae, and so provided a more readily interpretable profile of potential semiochemicals present in the wax layer than does solvent extraction. We tested this hypothesis by comparing hydrocarbon profiles of female *M. caryae* by the two sampling methods. *Z*9:C_{29} was the only compound among the dominant hydrocarbons that was present in higher abundance in SPME than in solvent extracts (~12% vs. ~8%, respectively), supporting this hypothesis.

Key Words—Cerambycidae, mating behavior, cuticular hydrocarbon, solid-phase microextraction, contact sex pheromone.

INTRODUCTION

The wax layer on the insect cuticle is a complex mixture of long-chain hydrocarbons, fatty acids, alcohols, esters, aldehydes, and ketones that protects insects from desiccation (Gibbs, 1998). Some of these compounds also have secondary roles as contact pheromones (Blomquist et al., 1993). Contact pheromones mediate mate recognition in several species of cerambycid beetles (Kim et al., 1993; Fukaya et al., 1996, 1997, 2000; Wang, 1998; Ginzel and Hanks, 2003; Ginzel et al., 2003a,b). We report here the results of studies on a cuticular sex pheromone of female *Megacyllene caryae* (Gahan) (Coleoptera: Cerambycidae).

M. caryae is endemic to north-central North America (Linsley, 1964). Adults are diurnal, aposematically colored wasp mimics, ~2 cm long, and are active in spring. Adults of both sexes are mutually attracted to volatiles emanating from their larval hosts, stressed or weakened hickory trees (Ginzel and Hanks, 2005). Males orient to females only after contacting them with their antennae, and mate recognition is cued by contact chemoreception (Ginzel and Hanks, 2003). After touching a live or freeze-killed female with his antennae, a male *M. caryae* aligns his body with that of the female and attempts to copulate, bending his abdomen to couple with the female's genitalia (Ginzel and Hanks, 2003). Males did not display any of these behaviors when presented with solvent-extracted, freeze-killed females, but reapplying crude solvent extract to solvent-washed female carcasses restored activity, demonstrating that mate recognition and subsequent mating behaviors are cued by extractable chemicals in the cuticular wax layer (Ginzel and Hanks, 2003).

We report here the identification of an important component of the female-produced contact sex pheromone for *M. caryae*. Two methods of sampling were used: the traditional whole-body solvent extraction and solid phase microextraction (SPME) wipe sampling. SPME has been suggested as an alternative to solvent extraction of insect cuticular hydrocarbons (e.g., Turillazzi et al.,

1998; Peeters et al., 1999; Liebig et al., 2000; Sledge et al., 2000; Roux et al., 2002) and reportedly yields samples that are qualitatively and quantitatively similar to those obtained by solvent extraction (Moneti et al., 1997; Monnin et al., 1998; Bland et al., 2001; Tentschert et al., 2002). However, study on the contact pheromones of a congener of *M. caryae*, *M. robiniae* (Förster) revealed that whole-body solvent extraction and SPME wipe sampling of female elytra, the most likely surfaces contacted by antennae of males, yielded markedly different hydrocarbon profiles (Ginzel et al., 2003b). The contact pheromone of female *M. robiniae*, *Z*9:C_{25}, comprised ~16% of the total hydrocarbons in hexane extracts of females and was codominant with two other hydrocarbons that were not active. In contrast, in SPME wipe samples of several areas of the cuticle, *Z*9:C_{25} appeared as the single dominant peak, comprising ~35% of the sampled hydrocarbons (Ginzel et al., 2003b). This finding suggested that hydrocarbons that cue mate recognition are more abundant on the surface of the cuticular wax layer in females where they are readily accessible to the antennae of males. We concluded that wipe sampling by SPME may yield a more representative profile of cuticular components actually encountered by the antennae of male insects, and so may provide a more representative profile of potential semiochemicals present on the body surface than does solvent extraction. We tested this hypothesis in the present study by sampling cuticular hydrocarbons of female *M. caryae* by both methods, predicting that compounds which cue mate recognition would be present in higher relative proportions in SPME wipe samples of the female cuticle than in whole-body extracts.

METHODS AND MATERIALS

Source of Beetles. The trunk of a shagbark hickory that was windthrown during spring 2002 at Brownfield Woods (Champaign County, IL) and infested with immature *M. caryae* was sectioned in March 2003 and the bolts stored in a 4°C walk-in cooler to prolong prepupal diapause. As adult beetles were needed for bioassays in May through August 2003, we moved logs to an indoor cage at ambient temperature to induce emergence. As adult beetles emerged, we housed individual males and females separately in 0.3 m^3 cages of aluminum window screen held under ambient laboratory lighting and temperature conditions. Caged beetles were provided feeder vials of 10% sucrose solution (glass vial with a cotton dental roll; Patterson Dental Supply, South Edina, MN, USA), and feeders were replaced every 2–3 d. Beetles used in preparation of extracts and in bioassays were vigorous and active in cages. Thirty adult male and female *M. caryae* that had emerged in 2003 were caged with shagbark hickory bolts (~30 cm diam, ~45 cm long) from a tree collected

at Allerton Park (Piatt County, IL, USA). Following oviposition, the bolts were stored at ambient temperature, and adults that emerged the following May were used in bioassays during 2004.

Preparation of Whole-body Extracts and Identification of Cuticular Hydrocarbons. Cuticular chemicals were extracted from 10 freeze-killed virgin female and male *M. caryae* by individually immersing each beetle in two 1-ml aliquots of hexane for 2 min each. The two aliquots were combined and concentrated to 1 ml under nitrogen. Extracts were initially analyzed at the University of Illinois at Urbana–Champaign (UIUC) by coupled gas chromatography-mass spectrometry (GC-MS) with electron impact ionization (EI, 70 eV) using a Hewlett-Packard (HP) 6890 GC (Hewlett-Packard, Sunnyvale, CA, USA) equipped with a DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm film; J&W Scientific, Folsom, CA, USA) in splitless mode, interfaced to an HP 5973 mass selective detector (MSD), with helium carrier gas. The column was programmed from 50°C/1 min, 10°C/min to 240°C, then 240 to 300°C at 2°C/min with a 5 min hold at 300°C. Injector and transfer line temperatures were 280°C. Quantitative data presented in Table 1 and Figure 1 were produced by these analyses.

Compounds in whole-body extracts were identified in the laboratory of J.G.M. with an HP 6890 GC equipped with a DB-5MS column (30 m × 0.25 mm × 0.25 μm film) and interfaced to an HP 5973 MSD. The temperature program was 100°C/1 min, 10°C/min to 280°C, with a hold at 280°C for 20 min. Injector and transfer line temperatures were 280°C. To determine the positions of double bonds in alkenes in the extracts, the crude extracts were epoxidized by treating an aliquot of an extract with a few drops of *m*-chloroperbenzoic acid in CH_2Cl_2 (25 μl of a 2 mg/ml solution). After 2 hr at room temperature, the mixture was extracted with 1 M aqueous NaOH, and the hexane layer was dried over anhydrous Na_2SO_4, and then analyzed as described above.

Solid Phase Microextraction. We sampled cuticular components of five adult female *M. caryae* by gently rubbing the dorsal surface of the elytra with a 100 μm (film thickness) polydimethylsiloxane SPME fiber (Supelco Inc., Bellefonte, PA, USA) for 30 sec. The same beetles also were sampled by whole-body hexane extraction, allowing us to compare profiles produced by the two sampling methods. Samples were analyzed at UIUC by GC-MS by desorbing the SPME fiber for 1 min in the injection port (250°C) with the same temperature settings that were used in analyzing extracts (see above).

Fractionation of Whole-Body Extracts. A column prepared from a Pasteur pipette plugged with a small plug of glass wool was loaded with 400 mg silica gel impregnated with $AgNO_3$ (10% w/w) and oven-dried overnight at 120°C. After cooling, the column was rinsed with hexane, then loaded with a concentrated composite extract of the whole-body extracts from six females in hexane (~0.5 ml total volume). The column was eluted sequentially with 4 ml hexane

(saturated hydrocarbons fraction), 2 ml 20% cyclohexene in hexane (alkenes fraction), and 3 ml ether (polar compounds). One half of the saturated alkanes fraction was concentrated to dryness under a stream of N_2, then 0.5 ml isooctane was added, and the extract was concentrated to dryness again to remove traces of hexane. The residue was taken up in 2 ml isooctane and stirred overnight with 50 mg oven-dried powdered 4 Å molecular sieve. The resulting slurry was filtered through a glass wool plug, yielding a branched alkanes fraction. The various fractions were tested for contact pheromone activity in bioassays.

Chemical Standards. $C_{27}-C_{29}$ straight-chain hydrocarbons were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Lancaster Synthesis (Pelham, NH, USA). The syntheses and spectral data of other standards, including *Z*9:C_{29}, *Z*13:C_{29}, *Z*14:C_{29}, *Z*13:C_{31}, *Z*14:C_{31}, *Z*15:C_{31}, 7Me-C_{25}, 11Me-C_{25}, 2Me-C_{26}, 9Me-C_{29}, 11Me-C_{29}, 13Me-C_{29}, 15Me-C_{29}, 13Me-C_{31}, and 15Me-C_{31}, are described in the online Supplementary Information available for this article, at http://dx.doi.org/10.1007/s10886-005-9010-y and is accessible for authorized users.

Bioassays with Fractions and Hydrocarbon Standards. Hexane solutions of hydrocarbon standards were prepared in concentrations that approximated the original extracts of female *M. caryae* (quantified by comparing peak areas from integrated total ion chromatograms with that of an internal standard). We tested the activity of the crude hexane extract of females, then the saturated hydrocarbon, branched alkane, and alkene fractions of the crude hexane extract, and then reconstructed the blends of the most abundant compounds based on chemical class. We then tested for synergistic responses of males to the monoenes, the most bioactive functional group (see Results), and the straight-chain alkanes. Next, we tested the bioactivity of the monoenes blend without *Z*9:C_{29}, the most abundant alkene in the hexane extract and SPME samples of the female cuticle (see Results). We then tested *Z*9:C_{29} alone and in combination with the straight-chain alkanes and methyl-branched alkanes (see Results). The bioactivities of these blends and individual compounds were tested with the following bioassay (see Ginzel et al., 2003a,b) in May–June 2003 and 2004:

(1) A female was freeze-killed (−4°C for 20 min), then the carcass was warmed to room temperature (~15 min), and presented to a male in a clean glass Petri dish lined with filter paper. An attempt to mate was taken as evidence that recognition cues were intact and that a behavioral response by the female was not necessary for mate recognition.
(2) We removed cuticular components from the dead female by immersing her in two sequential 1-ml aliquots of analytical-grade hexane for 2 min each.
(3) The solvent-washed female carcass was air-dried for 30 min to allow the solvent to evaporate and then presented to the same male. Lack of a

TABLE 1. CUTICULAR HYDROCARBONS OF FEMALE AND MALE *Megacyllene caryae*

Peak number	Retention time	Hydrocarbon	Percent of total hydrocarbons ± 1 SE[a]			Diagnostic Ions
			Hexane extracts		SPME	
			Male	Female	Female	
1	27.79	2Me-C_{26}	0.19 ± 0.001	13.2 ± 1.41	12.0 ± 1.19	380 (M^+), 365, 337
2	28.51	*n*C_{27}	0.39 ± 0.002	7.46 ± 0.84	5.49 ± 0.62	380 (M^+)
3	29.15	11Me-C_{27}	nd	2.75 ± 0.60	2.60 ± 0.96	379 (M^+-15), 168/252
		13Me-C_{27}				379 (M^+-15), 196/224
4	29.22	9Me-C_{27}	nd	1.07 ± 0.37	1.74 ± 0.65	379 (M^+-15), 140/280
5	29.73	2Me-C_{27}	nd	0.91 ± 0.10	1.38 ± 0.28	394 (M^+), 379, 351
6	30	3Me-C_{27}	1.09 ± 0.003	2.52 ± 0.35	2.86 ± 0.36	394 (M^+), 379, 365
7	30.56	*n*C_{28}	nd	1.15 ± 0.05	0.59 ± 0.06	394 (M^+)
8	31.92	2Me-C_{28}	6.30 ± 0.006	17.3 ± 1.32	12.5 ± 0.75	408 (M^+), 393, 365
9	32.05	*Z*13:C_{29}[b]	nd	3.36 ± 0.47	5.11 ± 0.56	406 (M^+), 83, 97, 111
		*Z*14:C_{29}[b]				406 (M^+), 83, 97, 111
10	32.26	*Z*9:C_{29}[b]	nd	7.73 ± 0.69	11.6 ± 1.11	406 (M^+), 83, 97, 111
11	32.77	*n*C_{29}	2.16 ± 0.003	9.57 ± 0.70	6.38 ± 0.66	408 (M^+)
12	33.48	11Me-C_{29}	0.09 ± 0.001	11.2 ± 1.16	12.1 ± 1.35	422 (M^+), 168/280
		13Me-C_{29}				422 (M^+), 196/252
		15Me-C_{29}				422 (M^+), 224
13	33.59	9Me-C_{29}	nd	3.45 ± 0.23	4.13 ± 0.27	422 (M^+), 140/308
14	34.11	13,17-, 11,15-, 9,13-dimethyl C_{29}	nd	1.76 ± 0.31	1.90 ± 0.18	421 (M^+-15), 196/267; 168/295, 224/239; 140/323, 252/211
15	34.51	3Me-C_{29}	8.66 ± 0.2	0.67 ± 0.25	0.35 ± 0.17	407 (M^+-15), 393
16	36.29	C_{31} diene	nd	0.35 ± 0.16	0.63 ± 0.19	432 (M^+), 82, 96, 110, 124, 138
17	36.42	C_{31} diene	nd	0.59 ± 0.11	1.01 ± 0.12	432 (M^+), 82, 96, 110, 124, 138

18	36.63	2Me-C_{30}	15.7 ± 0.02	4.27 ± 1.60	2.01 ± 0.35	436 (M^+), 421, 393
19	36.76	*Z*13:C_{31}[b]	nd	2.51 ± 0.32	3.75 ± 0.27	434 (M^+), 83, 97, 111
		*Z*14:C_{31}[b]				434 (M^+), 83, 97, 111
		*Z*15:C_{31}[b]				434 (M^+), 83, 97, 111
20	37.04	*Z*9:C_{31}[b]	15.7 ± 0.03	1.81 ± 0.25	2.90 ± 0.33	434 (M^+), 83, 97, 111
21	37.52	*n*C_{31}	4.34 ± 0.004	0.93 ± 0.15	0.90 ± 0.10	436 (M^+)
22	38.29	13Me-C_{31}	6.57 ± 0.003	4.14 ± 0.29	5.32 ± 0.54	435 (M^+-15), 196/280
		15Me-C_{31}				435 (M^+-15), 224/252
23	38.94	13,17-Dimethyl C_{31}	nd	1.35 ± 0.22	1.84 ± 0.23	451 (M^+-15), 196/295, 224/267
24	39.59	3Me-C_{31}	3.08 ± 0.003	nd	nd	435 (M^+-15), 435, 421
25	41.07	$C_{33:1}$[b]	3.30 ± 0.007	nd	nd	462 (M^+), 83, 97, 111
26	42.02	$C_{33:1}$[b]	4.58 ± 0.008	nd	nd	462 (M^+), 83, 97, 111
27	43.56	13Me-C_{33}	7.91 ± 0.004	nd	nd	463 (M^+-15), 196, 308
		15Me-C_{33}				463 (M^+-15), 224, 280
		17Me-C_{33}				463 (M^+-15), 252
28	44.17	13,17- & 15, 19-dimethyl C_{33}	10.3 ± 0.02	nd	nd	477 (M^+-15), 196/323, 252/267
						477 (M^+-15), 224/295
29	47.04	C_{35} diene	5.58 ± 0.007	nd	nd	488 (M^+), 82, 96, 110, 124, 138

[a]Peak numbers correspond to those in Figure 1. DB-5 MS capillary column, 50°C/1 min, 10°C/min to 240°C at, then 240–300°C at 2°C/min, 5 min hold at 300°C. Percent of total hydrocarbons represent means for five individuals. "nd" = Not detected.
[b]Double bond position and stereochemistry determined from mass spectra and retention times of the corresponding epoxides, see Results section.

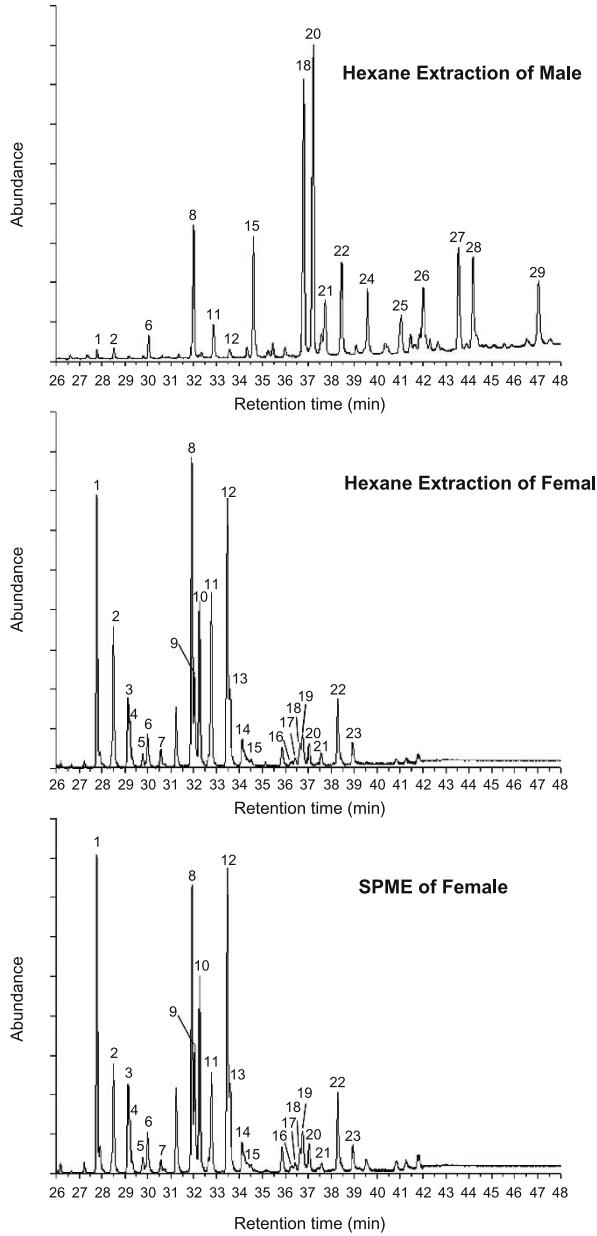


FIG. 1. Representative total ion chromatograms of whole-body hexane extracts of an adult male (top) and female (middle) *Megacyllene caryae*, and SPME wipe sample of female cuticle (bottom). Analysis conditions are described in Methods and Materials.

response by the male was evidence that chemical recognition cues had been eliminated.

(4) We tested the bioactivity of extracts, fractions of extracts, and individual or blends of hydrocarbon standards by pipetting solutions onto the body of the solvent-washed female, allowing the solvent to evaporate, and presenting the treated carcass again to the same male. We used 0.8 female equivalents (FE) in 20 μl hexane, because males responded best to this dosage of crude extract (Ginzel and Hanks, 2003). Test solution was applied to the solvent-washed female carcass and 20 μl hexane were applied to another solvent-washed female carcass as a control. The two carcasses were then presented to individual males simultaneously, on opposite sides of a Petri dish arena, with their positions randomized to control for location effects.

For all bioassays, we presented each of at least five dead females to two or three different males (N = 10–20 males). We videotaped responses of males in the Petri dish arenas. Males used in bioassays had not mated for 24 hr prior to bioassays and were used in bioassays only once per day. After a male *M. caryae* contacted a living female with his antennae, a clear progression of behaviors led to copulation: (1) the male oriented to (turned toward) the female, (2) the male stopped walking (= arrestment), (3) the male aligned his body with the female, and (4) the male mounted the female and attempted to couple the genitalia. Thus, our assessment of behavioral response was cumulative: males must perform steps 1 through 3 to reach step 4.

A trial was scored as a "response'' if the test male displayed at least the first behavior (turning toward female), within 5 min after first contacting a treated or control female carcass with his antennae. A trial was scored as "no response'' if the male showed none of these behaviors within 5 min of initial antennal contact, but rather continued to walk after contacting either a treatment or a control with his antennae. Statistical significance of the response of males to treatments was tested with Fisher's exact test (Sokal and Rohlf, 1995), (a) comparing numbers responding to crude extract (i.e., reaching behavioral step 1 at least) vs. solvent controls, (b) comparing numbers responding to treatments vs. crude extract, and (c) comparing numbers showing a complete mating response (i.e., reaching behavioral step 4) to treatments vs. crude extract.

RESULTS

Preliminary Bioassays. As a prelude to further investigation, we determined that male *M. caryae* could discriminate between female and male conspecifics by contact chemoreception, by pairing males with females and males with males in Petri dish arenas (N = 10 for each combination of sexes). Every

male attempted to mate with the female immediately after first antennal contact, but males only briefly antennated other males, then subsequently avoided them. The responses of male *M. caryae* to females and other males were consistent with earlier laboratory studies (Ginzel and Hanks, 2003; M.D. Ginzel, personal observation). These findings suggested that the chemical cues that males use to recognize females are absent in males, or that males have other compounds that inhibit the mating response.

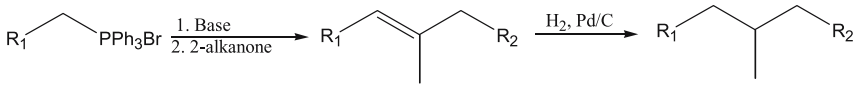
Identification of Cuticular Hydrocarbons. Hexane extracts of female and male *M. caryae* contained saturated, branched, and unsaturated hydrocarbons (Table 1, Figure 1). Cuticular hydrocarbon components were identified by comparison of mass spectra and retention times with those of standards, or from the parent M^+ ions and the corresponding molecular formulae, retention times relative to straight-chain compounds, and diagnostic mass spectral fragments that unequivocally demonstrated the position of methyl branches, as thoroughly documented by previous researchers (e.g., Nelson, 1993; Nelson and Blomquist, 1995). Double bond locations and geometries of monoenes were determined by epoxidation of extracts followed by GC-MS analysis of the resulting derivatives. The epoxides gave large diagnostic fragments from cleavage on either side of the epoxide, unequivocally demonstrating the position of the double bond in the parent molecule. GC-MS analysis of the epoxides of *E* and *Z* isomers determined that the *E* isomers eluted before the *Z* isomers on the DB-5MS column.

There were consistent, sex-specific differences in the hydrocarbon profiles of males and females, with the dominant compounds in solvent extracts of females including 2Me-C_{26} (peak 1), 2Me-C_{28} (peak 8), and coeluting 11-, 13-, and 15Me-C_{29} (peak 12). Extracts of males contained some of the compounds present in females as minor components, but also several longer chain hydrocarbons that were specific to males, primarily alkenes and mono- and dimethyl-branched alkanes.

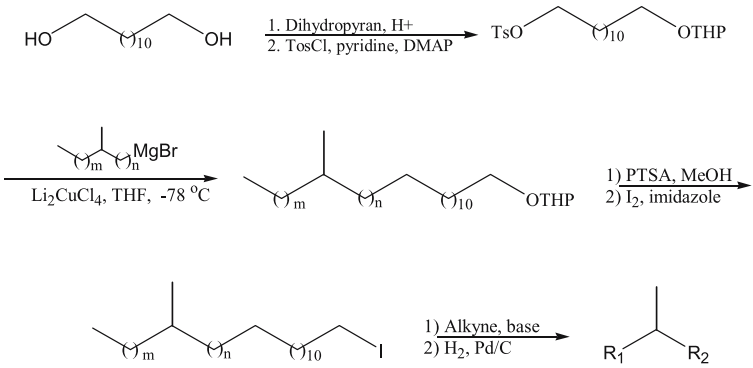
Solid Phase Microextraction. Samples of cuticular hydrocarbons from female *M. caryae* taken by SPME wipe sampling yielded a profile of hydrocarbons that was similar to that of the whole-body solvent extracts (Table 1; Figure 1 middle and bottom). All components present in SPME samples were also present in solvent extracts. The most conspicuous difference between profiles from solvent extraction and SPME sampling was a 50% increase in the relative amount of *Z*9:C_{29} in SPME samples (peak 10, Table 1; Figure 1, middle and bottom; means for two sampling methods significantly different, ANOVA: $F_{1,9} = 8.85$, $P < 0.018$). *Z*9:C_{29} was the only one of the dominant hydrocarbons that showed this increase in relative abundance, suggesting that it might be an important component of the contact pheromone of females.

Synthesis of Cuticular Hydrocarbons. Two strategies were used for the synthesis of monomethyl-branched hydrocarbons. The first strategy used Wittig

Route A:

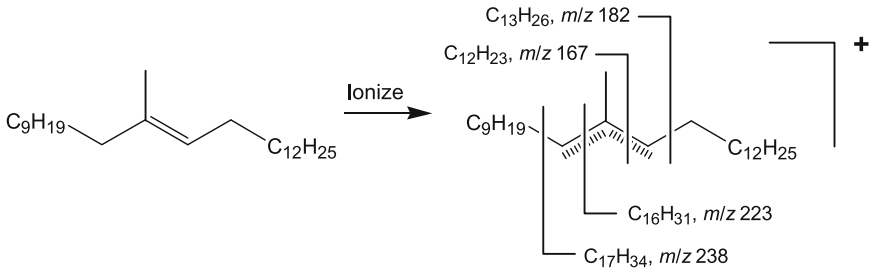


Route B: Building blocks synthesis



SCHEME 1. Synthetic routes for the preparation of monomethyl branched hydrocarbons.

reaction between a 2-alkanone and the appropriate phosphonium salt to generate methylalkenes, followed by catalytic hydrogenation (Scheme 1, route A). The commercial availability of many 2-alkanones (varying from acetone to 2-octadecanone) and *n*-alkylphosphonium salts rendered this two-step methodology simple and straightforward. Eight methyl-branched hydrocarbons were obtained using route A (Scheme 1), with overall yields varying from 38% to 65% (for details, see online Supplementary Information available for this article, at http://dx.doi.org/10.1007/s10886-005-9010-y and is accessible for authorized users). In the first step, the Wittig reaction was nonselective, producing the methylalkenes as mixtures of the *Z* and *E* isomers (ratios, 54:46 to 64:36). The EI mass spectra of these alkenes were characterized by distinct molecular ions (ca. 5–12% of the base peak) and two pairs of enhanced fragment ions, with ions in each pair separated by 15 mass units (Scheme 2). These ions indicated the position of the methyl group in the parent compound, but did not indicate which side the double bond was on. Catalytic hydrogenation of the methylalkenes then produced the desired methyl-branched alkanes, the structures of which can be unambiguously determined by EI mass spectrometry from the small molecular ions, and the enhanced even-mass ions from cleavage



SCHEME 2. EI-mass spectral fragmentation trends for monomethyl branched alkenes. Fragments from 11-methyl-11-pentacosene are illustrated.

on either side of the methyl group with a hydrogen transfer. A small M-15 fragment also was usually observed in the spectra, helping to confirm the M^+ ion.

The second synthetic route was designed to provide a flexible synthesis of compounds for which intermediates were not commercially available (Scheme 1, route B). This strategy is illustrated by the synthesis of 2Me-C_{26}. Thus, a double-ended building block was used, with introduction of the methyl branch at the desired position at one end by coupling a branched alkylmagnesium bromide and an O-protected tosylate with dilithium tetrachlorocuprate catalysis (Burns et al., 1997; Krause and Gerold, 1997). This was followed by deprotection of the other end of the building block and elongation of the chain to the desired length. Our first attempt at chain elongation by oxidation of 15-methylhexadecan-1-ol to the corresponding aldehyde, to be followed by Wittig reaction, failed due to polymerization of the aldehyde. Instead, the alcohol was converted to the iodide, followed by coupling with an alkyne (Corey et al., 1983) and reduction (Scheme 1, route B). Overall, this general strategy is amenable to production of a branched alkane with the branch in any desired position, and any desired chain length. It would also be amenable to the production of chiral hydrocarbons by attachment of a chiral rather than a racemic fragment in the first coupling step.

Bioassays with Fractions and Hydrocarbon Standards. All male *M. caryae* (100% of 200 trials) attempted to mate with freshly freeze-killed females, confirming that mate recognition cues were intact on female carcasses. Males did not respond to dead females after solvent extraction, consistent with earlier studies (Ginzel and Hanks, 2003; Ginzel et al., 2003a,b) and demonstrating that the contact sex pheromone(s) had been removed. None of the males (0 of 200 trials) showed any response to solvent-extracted dead female carcasses treated with pure solvent (controls) that were paired with hydrocarbon-treated females (Fisher's exact test of comparison with response to crude extract, $P < 0.001$).

TABLE 2. RESPONSE OF MALE *Megacyllene caryae* TO SOLVENT-WASHED DEAD FEMALES TO WHICH HYDROCARBON EXTRACTS OR STANDARDS HAD BEEN APPLIED IN DOSAGES OF 0.8 FE

Compound(s)	No. of males tested	Percent of males responding per step in behavioral sequence[a]			
		Step 1[b]	Step 2	Step 3	Step 4[c]
Crude extract	20	100	100	65	40
Saturated hydrocarbons fraction	10	60**	50	50	30
Branched alkanes fraction	20	40***	20	10	10*
Alkenes fraction	20	60**	60	30	10*
Straight-chain alkanes (*n*C_{27}, *n*C_{28}, *n*C_{29})	20	75*	65	15	0**
Branched alkanes (2Me-C_{26}, 9, 11, 13, 15Me-C_{29})	10	80	70	0	0*
Monoenes (*Z*9:C_{29}, *Z*13:C_{29}, *Z*14:C_{29}, *Z*13:C_{31}, *Z*14:C_{31}, *Z*15:C_{31})	20	100	95	25	25
Monoenes + straight-chain alkanes	10	80	80	30	20
Monoenes minus *Z*9-C_{29}	20	80	75	10	0**
*Z*9:C_{29}	20	95	90	35	25
*Z*9:C_{29} + straight-chain alkanes	20	100	95	40	15
*Z*9:C_{29} + branched alkanes	20	100	85	20	10*

Behavior of responding males was categorized according to the natural sequence of mating behavior (see Methods and Materials). Males did not respond to any of the solvent-washed controls.

[a]Responses of males to treatments vs. crude extracts were tested with Fisher's exact tests (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

[b]Number of males responding to treatments as measured by reaching behavioral step 1 was compared to responses to crude extract (H_0 = treatment and crude extract do not differ in activity).

[c]Number of males reaching behavioral step 4 in response to a treatment was compared to the response to crude extract (H_0 = treatment and crude extract do not differ in activity).

All of the males ($N = 20$) responded to females treated with 0.8 FE of their own crude extract, although only 40% reached behavioral step 4 (mounting the female and attempting to couple the genitalia; Table 2). Males showed a weaker response in behavioral step 1 to fractions (saturated hydrocarbons, branched alkanes, alkenes) of crude solvent extract of female cuticle when compared to their response to crude extract (Table 2), but the proportion of males that reached behavioral step 4 was similar for the saturated fraction and crude extract (Table 2).

We further evaluated the activity of structural classes by testing blends of synthetic components (Table 2). In these bioassays, the number of males re-

sponding (behavioral step 1) to a reconstructed blend of straight-chain alkanes was lower than the response to crude extract, as was the number of males reaching behavioral step 4 (Table 2). A reconstructed blend of branched alkanes did not differ from crude extract in bioactivity for the first step of the behavioral sequence, but no males reached behavioral step 4 (Table 2). Males responded most strongly to the reconstructed blend of the dominant monoenes, and addition of the straight-chain alkanes did not significantly affect activity (Table 2). Similar numbers of males reached behavioral step 4 in response to the blend of monoenes compared to crude extract (Table 2). However, activity of the alkene blend was reduced (no males reaching step 4) when *Z*9:C_{29} was eliminated. When tested separately, *Z*9:C_{29} elicited a response from males that was equivalent to that elicited by the complete blend of monoenes. Combining *Z*9:C_{29} with the blend of alkanes again did not affect the level of response compared to that elicited by *Z*9:C_{29} alone (Table 2). Finally, combining *Z*9:C_{29} with the dominant methyl-branched alkanes reduced its activity with respect to behavioral step 4 (Table 2).

DISCUSSION

Identification and Bioassay of Cuticular Hydrocarbons. Recent research on several longhorned beetle species in the subfamily Cerambycinae has suggested that mate location and recognition involves three distinct behavioral stages (Ginzel and Hanks, 2005): (1) the sexes are mutually attracted to volatiles emanating from host plants, (2) males on host plants produce volatile pheromones that draw females into their proximity over short or moderate distances (reviewed by Lacey et al., 2004), and (3) males recognize females by contact chemoreception with the antennae (see Ginzel and Hanks, 2003; Ginzel et al., 2003a,b). In the study reported here, the lack of response by male *M. caryae* to solvent-extracted female carcasses, or dead females treated with solvent alone, indicated that contact pheromones were the primary signals used for mate recognition, with tactile or visual cues possibly playing a subordinate role. The activity of *Z*9:C_{29} in bioassays indicated that it is an important component of the contact sex pheromone of female *M. caryae*, if not the sole component. Combinations of this compound with other cuticular chemicals failed to identify any synergists. Furthermore, *Z*9:C_{29}, is a homolog of *Z*9:C_{25}, the contact sex pheromone of the congener *M. robiniae*. The structural similarity in their contact pheromones is not unexpected given their close phylogenetic relationship. In fact, females of the two species share 13 cuticular hydrocarbons, including *Z*9:C_{29} (representing ~1% of the total hydrocarbons of female *M. robiniae*), and another seven of the 30 cuticular hydrocarbons of female *M. caryae* have chain-length analogs in *M. robiniae* (Ginzel et al.,

2003b). Other insect congeners, such as species in the *Drosophila melanogaster* group (Coyne, 1996), are known to share cuticular hydrocarbon components.

The role of *Z*9:C_{29} in mate recognition was further supported by its complete absence in males. In fact, extracts from male and female *M. caryae* were qualitatively very different, with sex-specific compounds representing almost half of the hydrocarbons of females and a third of the hydrocarbons of males (Table 1, Figure 1). Extracts from males contained a greater percentage of higher molecular weight, longer chain compounds, as appears to be true for some longhorned beetle species (Ginzel et al., 2003a,b). However, in other cerambycids, including *M. robiniae*, hydrocarbon profiles of males and females are qualitatively more similar, with most components being present in extracts from both sexes (Ginzel et al., 2003a,b).

The male-specific compounds of *M. caryae* may serve as abstinons (chemical deterrents to mating), although this function has yet to be investigated in cerambycids. Other types of insects, such as tsetse fly (Nelson and Carlson, 1986) and the bark beetle *Ips lecontei* Swaine (Page et al., 1997), have sex-based differences in alkyl chain lengths of cuticular hydrocarbons, but with higher molecular weight compounds predominating in females rather than males.

The fact that male *M. caryae* frequently did not exhibit mating behaviors toward solvent-extracted female carcasses that had been treated with their own crude extracts was unexpected, because males of other cerambycid species typically show strong responses to female carcasses treated with extracts of females in identical bioassays (e.g., Ginzel et al., 2003a,b). Males of a congener, *M. robinae*, may respond more strongly to reconstituted wax layers of females than male *M. caryae* because the contact pheromone was a dominant hydrocarbon in crude extracts (Ginzel et al., 2003b), while that of *M. caryae* was considerably less abundant.

*Z*9:C_{29} was not as dominant in hydrocarbon profiles determined by SPME of female *M. caryae* as was the contact pheromone of female *M. robiniae* (Ginzel et al., 2003b). Nevertheless, examination of Figure 1 revealed that *Z*9:C_{29} was the only compound among the dominant hydrocarbons that was present in higher abundance in SPME wipe samples than in solvent extracts. These findings support the hypothesis that identification of contact pheromones may be facilitated by comparing hydrocarbon profiles of solvent extracts and SPME, to determine which compounds are present in higher abundance on the surface of the wax layer (SPME) as opposed to throughout the wax layer (solvent extracts).

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